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ON THE TOXIC PROPERTIES OF SOME COPPER COMPOUNDS WITH SPECIAL REFERENCE TO BORDEAUX MIXTURE.

JUDSON F. CLARK.

(WITH SEVEN FIGURES)

WITHIN recent years copper has come to be the most valued agent in combating the ravages of plant diseases. Its importance from an economic standpoint has attracted to its study an unusually large number of investigators, and as a result there is a very extensive literature on the toxicology of copper compounds. The object of the present study was to determine more exactly the concentration of the various copper compounds required to inhibit germination or to kill the spores of a fairly large and quite representative selection of fungi grown under favorable conditions; to determine the effect of various media on the toxicology of copper; and to throw light on the toxicology of the Bordeaux mixture. I shall not attempt to review the literature of the subject, except to refer very briefly from time to time to a few studies which are of special interest in connection with points in the present study. Those especially interested will find work before 1896 quite carefully reviewed by Fairchild,[1] Swingle,[2] and Evans.[3] References and reviews of work since that time may be found in the various reviews. Hollrung's Jahresbericht[4] is especially useful in this connection.

[1] FAIRCHILD, D. G.: Bordeaux mixture as a fungicide. Bull. no. 6. Div. Veg. Path. U. S. Dept. Agric. 1894.

[2] SWINGLE, WALTER T.: Bordeaux mixture, its chemistry, physical properties, and toxic effects on fungi and algae. Bull. no. 9. Div. Veg. Phys. and Path. U. S. Dept. Agric. 1896.

[3] EVANS, WALTER H.: Copper sulphate and germination. Bull. no. 10. Div. Veg. Phys. and Path. U. S. Dept. Agric. 1896.

[4] HOLLRUNG, M.: Jahresbericht über die Neuerungen und Leistungen auf dem Gebiete des Pflanzenschutzes. Berlin. 1898 and 1899.

Copper occupies an intermediate position in regard to toxic value for the mold fungi.[5] It is surpassed more than a hundredfold by mercuric chlorid, silver nitrate, and potassium chromate and bichromate. It is a little less toxic than nickel and a little more so than cobalt; distinctly more toxic than iron and the mineral acids; twelve times as toxic as zinc; and about eighty times as toxic as ethyl alcohol. Its great superiority as a fungicide lies in its cheapness, the tenacity with which its hydroxid, when properly prepared, adheres to the foliage of plants, and, as I shall show later, the solubility of its hydroxid in the presence of or by means of substances found alike in the cell sap of the host plant and of the parasite, but more especially the latter.

For a detailed description of the method of using the Van Tieghem cell for work of this kind the reader is referred to my article in a former issue of this journal.[6] I may add that the method has proved eminently satisfactory for various physiological and toxicological studies with pure cultures of fungi, yeasts, and bacteria.

Precautions for sterilizing were observed throughout the study, except with a few cultures of hymenomycetes. The spores used, of such fungi as can be normally matured in artificial culture, were taken from pure cultures frequently renewed to insure high vitality. Rhizoctonia and currant cane blight cultures were inoculated with mycelium taken from fresh pure cultures. With Hypholoma and Coprinus,[7] spores taken from plants growing under natural conditions were used. Naturally, every precaution was taken to avoid contamination, and on the whole very little difficulty was experienced on this account.

It is very important in any quantitative study of this kind to have as nearly uniform a quantity of spores in the various cultures compared as possible. It is equally important to avoid bunches of spores in making the inoculation. Oversight in regard to either of these points is bound to result in more or less erratic and unreliable results. This is due to the fact that a toxic agent like copper, which effects the death of the spores by uniting with and precipitating the protoplasm, is itself also precipitated in the process and can no longer exert a deleterious influence. This precipitation process is naturally a strictly quantitative matter, and the amount of copper sufficient to sterilize a culture containing fifty spores may prove entirely inadequate were the number of spores quadrupled or even doubled. When bunches of spores are present the effect is still more mischievous. These bunches are relatively slowly penetrated by the copper-containing solution. Should the bunch contain air, as is usually the case, the process of penetration of the copper to the innermost spores is still further retarded. In the meantime such spores as are floating singly are quickly killed, precipitating their share of the copper, and it may be that long before the solution has fully penetrated the bunch it may have lost the greater part of its soluble copper and may be no longer able to kill the remaining spores. These may then germinate and grow almost as well as if no poison had been present. The difficulty was entirely overcome by soaking the spores for a longer or a shorter time in sterile distilled water with an occasional shaking. In some cases this soaking may be continued with advantage for days, in which case it is well to keep them at a low temperature to prevent germination. In all cases it was advisable to inoculate from a mixture of spores in water, a uniform inoculation being approximated in that way without great difficulty. It is equally important to have the culture drop of as uniform a size as possible. Absolute precision in these matters, however, is impossible, and the reader is reminded that slight variations in all cultures are to be expected. Indeed, as has been often suggested, spores taken from the same sporangium and placed in the same culture under absolutely similar conditions vary very markedly in their capacity to resist poisons and other unfavorable conditions.

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[5] CLARK, J. F.: On the toxic effect of deleterious agents on the germination and development of certain filamentous fungi. Bot. Gaz. **28**: 289–327, 378–404. 1900.

[6] CLARK, J. F.: Bot. Gaz. **28**: 293. 1900.

[7] I beg to acknowledge my indebtedness to Dr. Margaret C. Ferguson, of Cornell University, who supplied me with the spores of Hypholoma and Coprinus, and to whose studies in the germination of the hymenomycetes I am indebted for the knowledge that they would be satisfactory for germination purposes in the media used.

I would emphasize the importance of considering the *point of inhibition of germination* rather than the *death point* in consulting these results. The former can be determined with ease and absolute certainty by direct observation under the microscope, the latter only by transferring the spores to a fresh culture free as possible from the deleterious agent, and determining their success or failure to germinate under these conditions. The unavoidable source of error here is the transference of more or less of the copper compound with the spores. This quantity is doubtless often sufficient to prevent the germination of spores which although greatly reduced in vitality may be still alive. The general result of such transfers made at varying intervals from twenty-four hours to ten days indicated that if the germination of the spores be *inhibited* by a concentration of a copper salt, it was merely a question of time when they would be killed. Thus if a certain concentration inhibited the germination of a certain spore, possibly a solution of four times this concentration would fail to sterilize the culture in twenty-four hours, yet if left for some days the points of inhibition and death would finally coincide. The length of time necessary to bring this about would naturally vary with the varying specific characteristics of the spores of the different species.

The medium used for the cultures, the results of which are given in *fig. 1*, was a decoction of sugar beet. The root, sliced thin, in the proportion of 450^{gr} per liter, was heated in an autoclave for an hour at 120° C., under a pressure of one atmosphere. The decoction was then filtered and poured into flasks and thoroughly sterilized. This medium was selected for the comparative tests as giving the best results with the greatest number of the fungi used. The other vegetable decoctions were prepared in essentially the same manner.

Stock solutions of the various copper compounds were prepared by a responsible chemist. A *normal* solution of a copper compound means in this paper the number of grams of the salt corresponding to the full molecular weight (*e.g.*, $CuSO_4.5H_2O=$ 2 49) dissolved in sufficient water to make one liter of solution; thus 0.01 *n* $CuSO_4$ equals a 0.249 per cent. concentration of the *crystallized* salt.

In the figures used to illustrate this paper the solid black bar indicates that the concentrations used were sufficiently strong to kill the spores within twenty-four hours. The square blocks alternating with the open spaces indicates that while the germination of the spores was inhibited, they still retained vitality enough to germinate several per cent. after twenty-four hours immersion. Four parallel lines indicate some germination but very poor development. Three parallel lines indicate fair but distinctly abnormal development, and two lines approximately normal germination and growth.

Fig. 1 shows in a condensed form the results of some four hundred cultures with fifteen fungi in a decoction of sugar beet to which had been added copper sulfate. A glance at the page will show that many of the fungi present a really remarkable similarity in resistance to copper. Twelve of the fifteen forms represent a range in lethal concentration of from 0.0099 *n* to 0.0168 *n*, or slightly less than 70 per cent. variation. This range of susceptibility may be modified by several important considerations, prominent among which is temperature. All, except Gloeosporium, were grown at 28° C., a little more or less. It is established that a fungus presents its greatest resistance to a deleterious agent, other things being equal, when cultivated at or near its optimum temperature for germination and mycelial development.[8] At a higher or lower temperature a smaller quantity of the agent results fatally. All the forms used, except *Gloeosporium malicorticus*, grew well at 28° C., but it is quite certain that some of them grow better at other temperatures. Inasmuch as the optimum temperatures for the different forms were unknown it was thought best to use a uniform

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[8] CLARK, J. F.: On the toxic value of mercuric chlorid and its double salts. Jour. Phys. Chem. 5: 289-316. 1901; also FLENDE: Ueber Creolin und seine Wirksamen Bestandteile. Arch. f. Hyg. 9: ———. 1889.

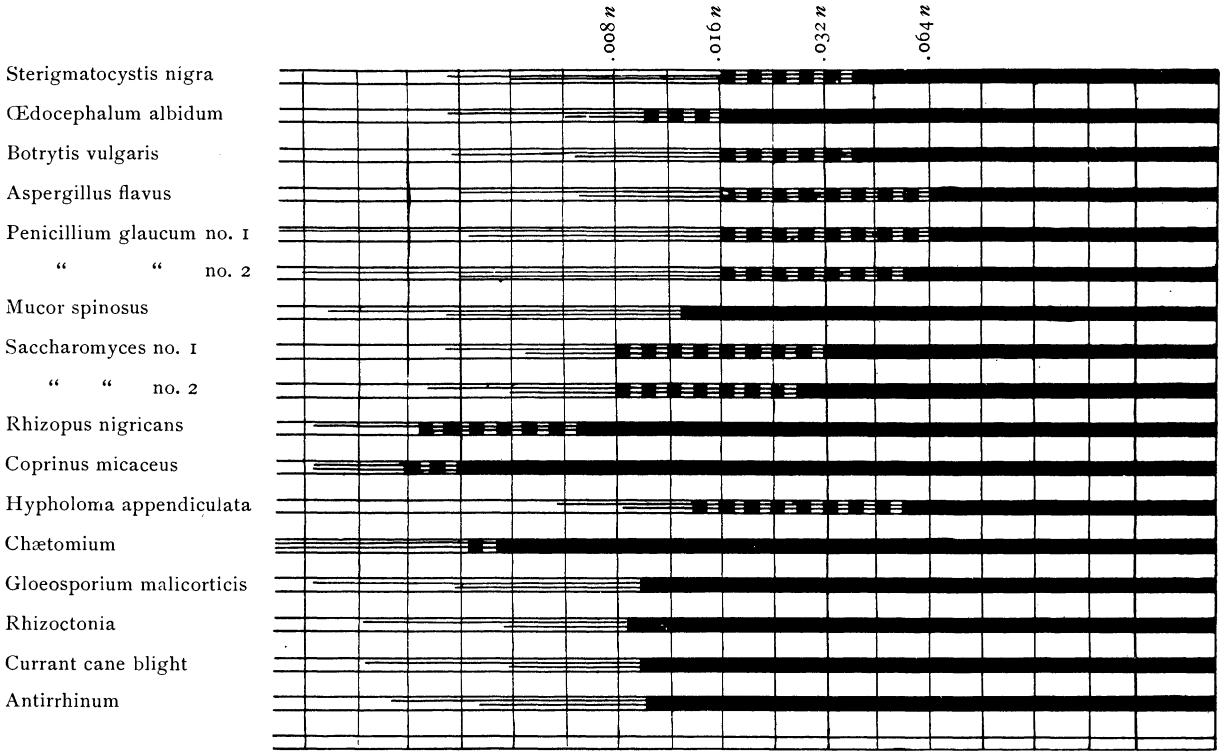

FIG. 1.

NOTE.—On account of the loss of the original drawings, while in the hands of the engravers, it is not possible to give *quantitative* values for this figure. The four values given are approximately correct. *Coprinus* was inhibited by .003 *n*.

temperature, except with Gloeosporium, which refuses to germinate at so high a temperature. It is also doubtless true that other *optimal* considerations have a similar effect in increasing the resistance of plants to unfavorable conditions. It should also be noted in studying these results that several of the forms were tested in media prepared at different times, and as sugar beets vary in composition the media would vary somewhat in nutritive properties, and therefore in toxicological properties also, when used as a solvent for a poison. Making due allowance for these considerations, however, it is perhaps remarkable that of a selection of fungi representing fairly well the natural groups and including typical saprophytic and parasitic forms, the great majority of them should be found to be killed under similar conditions by concentrations of copper of between 0.0099 *n* and 0.0168 *n*, and that the three forms which were found to be markedly more sensitive (Rhizopus, Coprinus, and Chaetomium) should be of such diverse relationships. The two species of yeasts proved to be equally resistant, although markedly different morphologically. The exact similarity of resistance was in this case very easily and prettily shown by growing both forms in the same culture drop. Such a test is applicable only where the forms are of similar properties as regards resistance to the agent, time of germination, and rapidity of growth. Two cultures of *Penicillium glaucum*, obtained from different sources and presenting slightly different habits of growth, also proved to be equally resistant.

The great variation between the two hymenomycetes is partly to be accounted for by the fact that the medium was very favorable for the development of Hypholoma, and only moderately suited for Coprinus. The similarity of resistance of the four parasitic forms (at bottom of figure) is quite striking. It will also be noted that they occupy an intermediate position. The most resistant forms will be seen to be the molds Aspergillus, Sterigmatocystis, Botrytis, and Penicillium.

Dr. Duggar has for some time been studying the broad field of food relation to toxicology in general. It has been ascertained that fungi vary greatly in their resistance to deleterious agents, according to variations in the medium in which they are cultivated when submitted to the influence of the agent. His results have not yet been published, but the importance of this matter in any toxicological studies necessitates considerable reference to the subject in this study of the toxicology of copper.

The differences in resistance due to variations in the culture media may be due to two distinct causes: (1) All media influence the toxicology of electrolytes, and possibly other agents, by causing certain physical and chemical changes in the condition of the dissolved agent; (2) other things being equal, the more favorable the medium for germination and early mycelial development of a fungus, the more resistant that fungus will be to deleterious agents.

The simplest case of changed physical or chemical conditions would be the diluting of the solution of the copper salt with pure water. Here, with each decrease in concentration, we get less and less of the whole molecule, *e. g.*, $CuSO_4$, and more and more of its component ions, $^{+}Cu^{+}$ and $^{-}SO_4^{-}$, and the amount of change may be readily determined for any particular dilution. When the dilution is accomplished by adding an infusion or decoction of a vegetable or animal substance, however, a series of unknown reactions occurs, and even the amount of copper in the ionic form cannot be known. That these transformations have a very great influence on the toxic properties of the mixture is abundantly shown by the results represented graphically in *fig. 2*. A number of media tested caused precipitation of the copper, thereby rendering it harmless.[9] All such media were discarded and are not represented in the figure.

When spores are placed in a medium which does not admit of growth (*e. g.*, pure water) it is merely a question of time when these spores will be killed, if there be present a sufficient quantity of copper to precipitate the living proteids. It takes some time, however, perhaps weeks, for all this copper to find its way into the spores. Should the medium be such as admitted growth, the production of protoplasm might easily offset its precipitation by the copper, and long before the copper could have its maximum effect the protoplasm would have increased manifold, and would require a corresponding increase in the concentration of copper to cause death. Thus it is that with every favoring influence the fungus can make a better fight for life.

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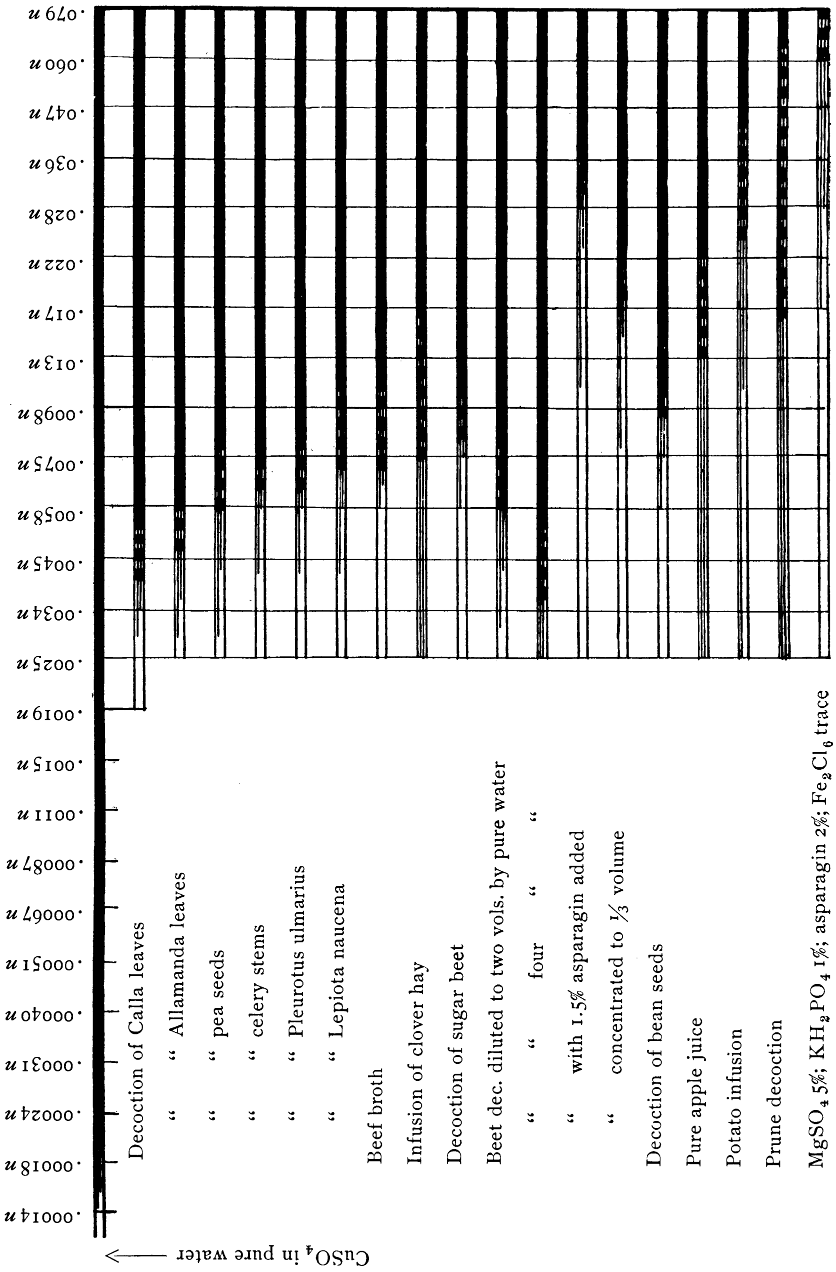
[9] COPELAND and KAHLENBERG: The influence of the presence of pure metals upon plants. Trans. Wis. Acad. **12**: 454–474. 1900.

The results given in *fig. 2*, with various media, and with copper in the form of the sulfate, are those with *Œdocephalum albidum*. The work repeated with *Rhizopus nigricans* gave essentially the same results. A single glance at the figure shows how much more toxic the copper proved to be when dissolved in pure water than when in any other medium, 0.0002 *n* being fatal in twenty-four hours. This is but one-fortieth of the concentration of copper required to give the same result in a decoction of sugar beet. When 1.5 per cent. asparagin was added to this beet infusion the toxic value of copper dissolved in pure water was to its value in the asparagin-beet medium as 142 : 1, and the comparison in the case of the medium compounded from asparagin and inorganic salts the ratio is 285 : 1. In this last case there was a slight precipitate noticed under the microscope, and it may be that some of the copper was precipitated by the phosphoric acid present. Exactly what proportion of this striking difference is to be assigned to the nutritive condition and what to chemical and physical transformations it is impossible to say. Inasmuch as the asparagin medium, while a good food for Œdocephalum, is not so good as beet decoction, we must concede that the remarkable decrease in toxic properties in the cases where asparagin is present is chiefly due to the physical and chemical changes. That these changes have a preponderating influence in the case of the beet decoction is also very probable. The experiment of diluting and concentrating the beet decoction seems to support this view. The values of the triple, the ordinary, the half, and the quarter strengths are not greatly different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.0025n
.0019n
.0015n
.0011n
.00087n
.00067n
.00051n
.00040n
.00031n
.00024n
.00018n
.00014n
Decoction of Calla leaves
" " Allamanda leaves
" " pea seeds
" " celery stems
" " Pleurotus ulmarius
" " Lepiota naucena
Beef broth
Infusion of clover hay
Decoction of sugar beet
Beet dec. diluted to two vols. by pure water
" " four " "
" with 1.5% asparagin added
" concentrated to 1/3 volume
Decoction of bean seeds
Pure apple juice
Potato infusion
Prune decoction
MgSO4 5%; KH2PO4 1%; asparagin 2%; Fe2Cl6 trace
CuSO4 in pure water

Fig.

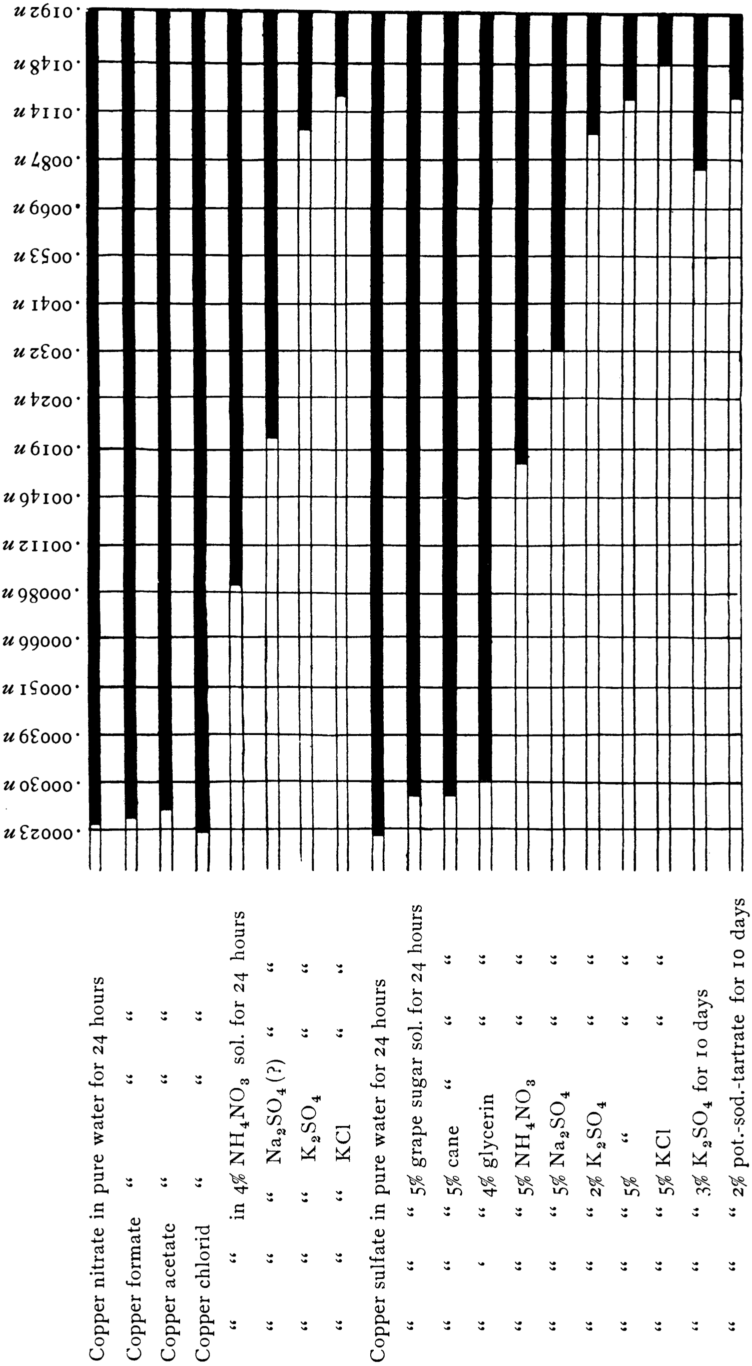
so far as stimulation of germination and early mycelial development is concerned. The same is true of the beet decoction to which the asparagin was added. Yet we have the following variation in the lethal concentrations:

Medium	Lethal concentration	Relative amount of Cu required to inhibit as compared with a pure water solution
Beet decoction, normal strength	0.0076 *n*	38 times
" " diluted to two volumes	0.0054 *n*	27 "
" " " " four "	0.0034 *n*	17 "
" " evaporated to ⅓ volume	0.0163 *n*	82 "
" " + 1.5 per cent. asparagin	0.0284 *n*	142 "

Were it wholly a matter of food relation we should expect the figures in the third column to be near 38 in each case. Were it wholly a matter of the chemical or physical transformation of the copper by the compounds present in the beet decoctions, we should expect a more proportionate decrease in the first three numbers of the third column, corresponding to the dilution of the medium by the added pure water. As already remarked, it is not possible to determine with any degree of precision the part played by any one factor from the data at hand; but it is evident that with different media great differences in the toxic value of the copper may be obtained, and that asparagin has the power of uniting with salts of copper in such a way as to reduce their toxic properties in a very extraordinary manner. It is of interest in this connection to note that mercuric chlorid is not similarly reduced in toxic properties by the presence of asparagin.[10]

Fig. 3 presents the results of some studies on the addition of certain compounds to pure water solutions of some copper salts. Here the results given are with Œdocephalum, and, as in *fig. 2*, the results were checked over with *Rhizopus nigricans*. In this diagram the vertical lines representing the variation in the concentration of copper present in the different cultures show an increase of 30 per cent. for each division over the last preceding

[10] Jour. Phys. Chem. 5: 302. 1901.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.0069 n
.0053 n
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.0032 n
.0024 n
.0019 n
.00146 n
.00112 n
.00086 n
.00066 n
.00051 n
.00039 n
.00030 n
.00023 n
Copper nitrate in pure water for 24 hours
Copper formate " " " " "
Copper acetate " " " " "
Copper chlorid " " " " "
" " in 4% NH_4NO_3 sol. for 24 hours
" " " Na_2SO_4 (?) " " "
" " " K_2SO_4 " " "
" " " KCl " " "
Copper sulfate in pure water for 24 hours
" " " 5% grape sugar sol. for 24 hours
" " " 5% cane " " " "
" " " 4% glycerin " " "
" " " 5% NH_4NO_3 " " "
" " " 5% Na_2SO_4 " " "
" " " 2% K_2SO_4 " " "
" " " 5% " " " "
" " " 5% KCl " " "
" " " 3% K_2SO_4 for 10 days
" " " 2% pot.-sod.-tartrate for 10 days

FIG. 3.

division passing to the right. It will be observed that the five copper salts, the nitrate, formate, acetate, chlorid, and sulfate, dissolved in pure water have approximately the same toxic value, the variation (0.00004 *n*) being within the limits of experimental error. These five salts are fatal to Œdocephalum in 0.00020–0.00024 *n* concentration in twenty-four hours. This is about a 0.005 per cent. solution of the crystallized salt, $CuSO_4.5H_2O$, or one part crystallized salt to 20,000 parts water.

The addition of glycerin, glucose, or cane sugar failed to cause any pronounced decrease in toxic properties. The addition of salts like K_2SO_4, KCl, etc., however, caused a very great reduction. One part $CuSO_4$ to 20,000 parts pure water is fatal to Œdocephalum in twenty-four hours. It requires sixty-one parts $CuSO_4$ to 20,000 parts of a 5 per cent. K_2SO_4 solution to have the same effect. That the lessening of the toxic value here was not due to a lessening of the rapidity of the penetration of the copper salt is shown by the last three lines of *fig. 3*, where the cultures after standing ten days showed a toxic value of less than one-fortieth that of the $CuSO_4$ in pure water. A test made by adding a small quantity of KCl to solutions of $CuCl_2$ gave an increase in the toxic value of the mixture. Further experimentation on this point is needed, but the indications are that here we have a similar phenomenon to that observed on the addition of chlorids to mercuric chlorid solutions,[11] small additions of a chlorid causing an increase, and larger amounts causing a decrease in toxic properties; the former probably through the formation of highly toxic $^-HgCl_4^-$ ions, and the latter through the formation of K_2HgCl_4 double salt molecules having a low toxic value. Copper reacts very similarly to mercury in respect to the formation of double salts with alkali chlorids.[12] These double salts would naturally be highly dissociated into metallic cations and the anion $^-CuCl_4^-$, and similar anions. That the ion $^-CuCl_4^-$ should have a high toxic value is very probable, being in harmony with all at present known regarding toxic agents capable of uniting with more chlorin.[13] That the forcing back of the ionization of these double salts to the molecular form (*e. g.*, K_2CuCl_4) would result in a lowering of its toxic value seems more than probable, in view of what we know in the analogous case of the double salt K_2HgCl_4 already referred to, and furnishes a very reasonable hypothesis to account for the remarkable lowering of the toxic value of solutions of copper in pure water when a chlorid is added in excess. The decrease from the adding of sulfates, nitrates, etc. of the alkali bases is doubtless due to similar conditions.

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[11] CLARK, J. F.: Jour. Phys. Chem. **5**: 289–316. 1900.

[12] REMSEN, IRA: Inorganic chemistry (advanced course) 589. 1892.

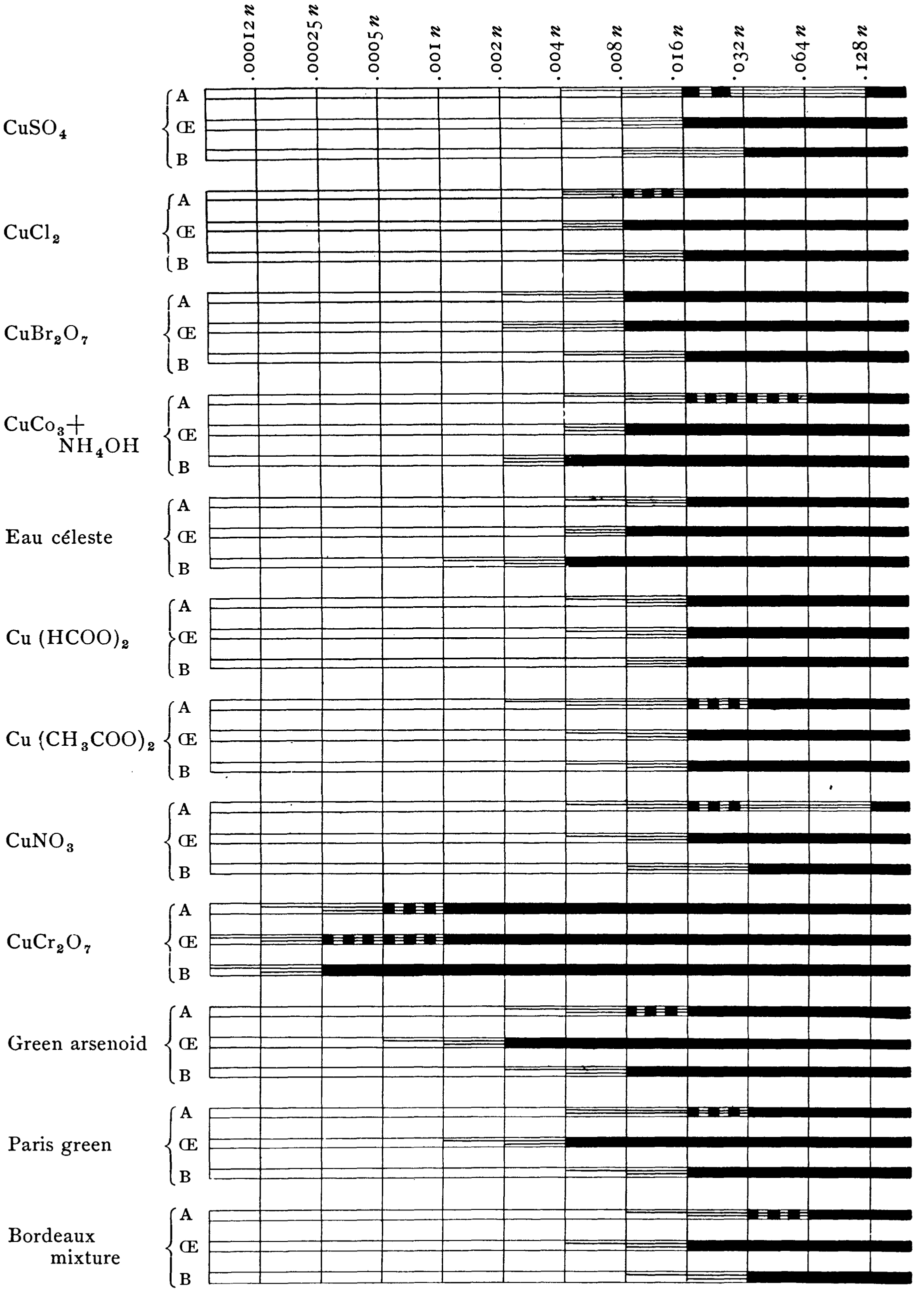
Fig. 4 gives the results of a test of various soluble and insoluble copper compounds with three fungi grown in beet decoction. The letters *A*, *Œ*, and *B* refer to Aspergillus, Œdocephalum, and Botrytis respectively. Beet decoction has a very marked power of dissolving copper even in the metallic state, hence it was possible to test "insoluble" copper compounds, such as Paris green, copper hydroxid (the form in which the copper is found in Bordeaux mixture), etc. This power of dissolving copper is a property of almost all decoctions of vegetable or animal substances, some possessing it in a much higher degree than others.

Fig. 4 shows that copper, gram for gram, has much the same value toxicologically when added to a beet decoction, no matter what form of copper salt is used. The apparent exceptions to this are are to be accounted for by the effect of the other atoms and groups added. Thus the chromate salt which proved to be by far the most toxic compound of copper tested owes its high toxic value to the deadly chromium contained.[14] The *eau céleste* and the ammoniacal solution of copper carbonate are particularly fatal to Œdocephalum and Botrytis on account of the basic alkalis present.[15] Œdocephalum is apparently very sensitive to compounds containing arsenic. Perhaps it is especially sensitive to this element, but so far as I know the point has not been tested. That the green arsenoid should be more fatal

[13] DAVENPORT, C. B.: Experimental morphology 8. 1897.

[14] CLARK, J. F.: Bot. Gaz. **28**: 390, 396. 1899.

[15] *Ibid.* **28**: 380–382. 1899.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.128 n
$CuSO_4$
A
Œ
B
$CuCl_2$
A
Œ
B
$CuBr_2O_7$
A
Œ
B
$CuCo_3$ + NH_4OH
A
Œ
B
Eau céleste
A
Œ
B
Cu $(HCOO)_2$
A
Œ
B
Cu $(CH_3COO)_2$
A
Œ
B
$CuNO_3$
A
Œ
B
$CuCr_2O_7$
A
Œ
B
Green arsenoid
A
Œ
B
Paris green
A
Œ
B
Bordeaux mixture
A
Œ
B

FIG. 4.

than the Paris green was to be expected, inasmuch as it showed on analysis 8.3 per cent. soluble As_2O_3 as compared with 1.8 per cent. soluble in the sample of Paris green. That the ammoniacal solution of $CuCO_3$ is a more effective fungicide than Bordeaux mixture for the amount of copper used does not follow from the showing made in this figure. These tests were made in closed cells where the excess of ammonia was indefinitely retained, whereas under orchard conditions this evaporates in a few hours at most, and exerts practically no fungicidal value.

It would be beyond the province of the present paper to give a detailed account of my experiments on the toxicology of the Bordeaux mixture. These are of greater interest to horticulturists than to botanists. I shall mention briefly, however, the more important experiments and state the conclusions arrived at from this study.

Just how the insoluble copper hydroxid of the Bordeaux mixture sprayed on leaves has protected them so thoroughly from the ravages of parasites has long been a mystery to the chemist and physiologist alike. Various hypotheses have been propounded from time to time, but up to the present no satisfactory explanation has been given. A very careful review of the current hypothesis may be found in Swingle's paper.[16] His own suggestion that it might be possible that the fungi themselves, by secretions or excretions, might be able to aid in the solution of the copper has proved a happy one. As already mentioned, most animal and vegetable decoctions have a greater or less power of dissolving insoluble copper compounds.

This power is particularly noticeable in the fungi. An infusion of *Agaricus campestris*, and infusions and decoctions of various parasitic fungi, dissolve copper hydroxid very quickly and in sufficient quantity to inhibit the germination of any fungus tested. Spores of various fungi placed in a mixture of $Cu(OH)_2$ and water were killed in a short time, while other spores of the same species, placed in similar mixtures of $Fe(OH)_2$

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[16] SWINGLE, W. T.: Bordeaux mixture. Bull. no. 9. Div. Veg. Phys. and Path. U. S. Dept. Agric. 1896.

and $Al(OH)_3$ retained their vitality unimpaired for weeks. The death of these spores was not due to the presence of any soluble copper, for no copper could be detected in the filtrate by the most delicate chemical tests before the spores were added. Traces of copper were found some time after the addition of the spores (if added in considerable quantity) showing that they dissolve more of the $Cu(OH)_2$ than was required to kill them. The rapidity with which fungus spores are thus killed naturally varies with the character of the contents and coverings of the spores. It is very quickly accomplished in thin-walled spores, such as Rhizopus and Œdocephalum, but is very slowly accomplished in forms like Aspergillus and Penicillium, whose coats present great resistance to the entrance of poisons and the excretion of copper-dissolving compounds. As an example of such resistance to entrance, see experiments with Aspergillus in solutions of nickel sulfate,[17] in which $0.03\,n$ inhibited germination, but immersion in a normal solution (containing over 13 per cent. $NiSO_4$) for 48 hours at 28° C. failed to kill the spores. The same form resists immersion in double normal $ZnSO_4$ (27 per cent. anhydrous $ZnSO_4$) under similar conditions.

The host plant, too, is active in dissolving the $Cu(OH)_2$. A peach tree was sprayed with Bordeaux mixture which contained an excess of lime, as is ordinarily the case. Following this, the tree was sprayed several times during the day with enough pure water to have an effect resembling dew. The following day water collected from the leaves, filtered and evaporated to small volume, showed just a slight copper reaction. Several small leafy branches were then removed and placed in a large beaker of distilled water, without injury to any of the parts. After soaking for some hours these were removed and the solution filtered, evaporated to small volume, and tested for copper. A marked reaction was secured, indicating the presence of considerable copper in solution. The epidermis of leaves, although protected by a cuticle, is well known to be more or less permeable to the dissolved substances occurring in the cell sap, particularly along the union of the epidermal cells.[18] When the dew is on the leaf we have two solutions—the dew drop without and the cell sap within—separated by a more or less permeable membrane. The result of these conditions must result in the exosmosis of at least some of the contents of the cell sap, which coming in contact with the copper hydroxid adhering to the leaf surface causes more or less of it to pass into solution. An important point in this connection is that the solution of the copper, whether effected by the host or the fungus spore itself, is possible in the presence of an excess of lime. This excess is apparently somewhat detrimental to the solvent action on the copper, and in this way doubtless serves a very valuable purpose in preventing a too rapid solution. It also protects the $Cu(OH)_2$ from the solvent action of the ammonia, nitrites, and nitrates of the atmosphere. According to Millardet and Gayon's[19] experiments, the amount of lime ordinarily used in Bordeaux mixture is sufficient to protect the copper absolutely from solution for eighteen days under ordinary atmospheric conditions. This ought to have been sufficient to have set aside the hypothesis which accounted for the solution of the copper of the Bordeaux mixture by atmospheric conditions, for all experiments go to show that the Bordeaux mixture is effective from the day it is applied. So much in brief review of some of the experiments that have led me to the following conclusions regarding the toxicology of this invaluable fungicide:

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[17] CLARK J. F.: Bot. Gaz. **28**: 392. 1899.

1. The solution of that part of the $Cu(OH)_2$ of Bordeaux mixture which under orchard conditions *is of fungicidal value, is chiefly accomplished by the solvent action of the fungus spores themselves*, for they have power to dissolve sufficient copper to kill themselves.

2. The amount of copper necessary for the destruction of the spores of parasitic fungi is probably not more than one part of soluble metallic copper to 80,000 parts water (dew).

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[18] BÜSGEN: Ueber einige Eigenschaften der Keimlinge parasitischer Pilze. Bot. Zeit. **57**: 67. 1893.

[19] MILLARDET et GAYON: Les divers procédé de traitements du mildiou par les composés cuivreux. Jour. des Agr. Prat. **1**: 701–702. 1887.

3. The host plant has a greater or less power of dissolving the $Cu(OH)_2$ deposited on its leaves.

This solution of the copper by the host plant may or may not be advantageous in protecting it from attack, as injurious spores may or may not chance to be present at the point where solution has taken place. To this factor, however, must be attributed the injury frequently observed with certain plants (*e.g.*, Japanese plums, peach, etc.) when sprayed with Bordeaux. The amount of injury done to a given species will depend on the following considerations, provided always that carefully prepared Bordeaux mixture has been used:

1. The *specific susceptibility* of the protoplasm of the plant to poisoning by copper.
2. The *solvent properties* of the cell sap on copper hydroxid.
3. The *permeability of the epidermis or cuticle*[20] to these cell contents when the conditions are favorable for their exosmosis, and for the entrance of the copper after its solution is effected.
4. Weather conditions following spraying, particularly conditions as regards moisture (dew, etc.), providing the conditions for the exosmosis of some of the contents of the cells of the leaf.

To these might be added, perhaps, climatic conditions which, through modifications of structure, of specific resistance, or of the character of the cell sap, may cause a plant to be more or less resistant to treatment with Bordeaux mixture, according to the locality where it is grown. The very injurious influence of Bordeaux mixture on blossoms[21] is to be explained in an entirely similiar way. Here the protection of the cells by cutinization is almost or entirely wanting, and it is very probable that the rich contents of pollen grains and the cells of the stigma have an unusually solvent action on the copper hydroxid.

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[20] Dr. W. C. Sturgis (Rep. Conn. Agric. Exper. Sta. 219–254. 1900) has done some very careful work on the effect of Bordeaux mixture on peach foliage. In his study for a cause of the extreme susceptibility which he found in his peach trees, he has unfortunately overlooked the possibility of specific sensitiveness to copper poisoning of the protoplasm; and in the study of the structure of leaves the study of the cutinization of the epidermis has been omitted. It is hardly possible that the aeration of the spongy parenchyma can be a factor in the case.

[21] BEACH, S. A., and BAILEY, L. H.: Fruit-setting of sprayed blossoms. Bull. 196. N. Y. Agric. Exper. Sta. 1900.

Swingle's proposition that the copper might be effective in preventing the attack of parasites through negative chemotactic action induced me to make a number of experiments on this point. A number of fungi were used to a greater or less extent, but Rhizopus proved the most satisfactory, inasmuch as it usually germinates with a single germ tube and grows rapidly with a minimum of branching.

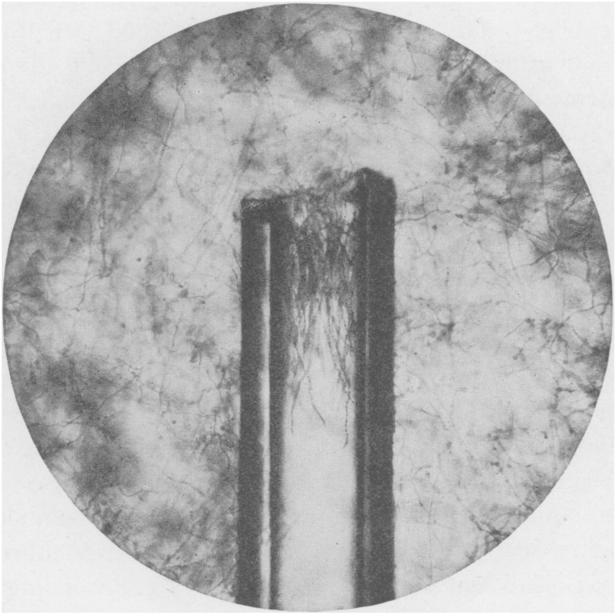

FIG. 5.

The well-known method of Pfeffer was first employed. Capillary tubes closed at one end were filled with solutions of copper of varying strengths. Hanging drop cultures of the fungi were prepared in weak agars of varying consistence. Into these hanging drop cultures were introduced the open ends of the copper-containing capillary tubes. These tubes were introduced at different stages of germination. The uniform result of these preparations was that the hyphae near the mouth of the tube grew into the solution of copper, and continued to grow directly inwards until they reached a concentration sufficient to kill them (*fig. 5*). This was found to be the case with copper, cobalt, nickel, zinc, etc., and *also when the capillary tube contained pure water.*

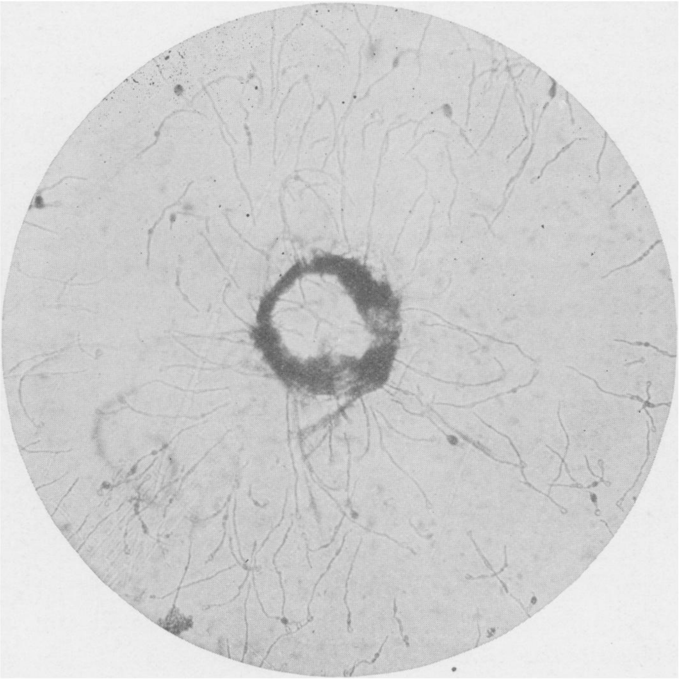

FIG. 6.

Miyoshi's[22] method of injecting leaves with solutions by placing them in the solutions under an air pump and exhausting was next tried. After the injection of the leaves they were washed under the faucet and spores of Rhizopus were sown on the lower surface. The preparation was then placed in a moist chamber in an incubator at 28° C. The germ tubes near stomata curved towards and grew directly into them in a manner quite similar to what Miyoshi found to be the case when the leaf was injected with food solutions. In many cases upwards of thirty germ tubes were counted growing into a single stoma. On injecting similar leaves with pure water, however, I got a similar result. The leaves used were chiefly from *Tradescantia discolor.* The concentrations of copper, cobalt, etc., injected ranged from 0.01 to 0.005 *n.*

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[22] MIYOSHI, M.: Ueber Chemotropismus der Pilze. Bot. Zeit. **52**: 1–27. 1894.

I then tried Miyoshi's method of using perforated mica plates with layers of media between. Spores were sown in every conceivable way: in upper, middle, and lower layers; in nutrient and non-nutrient media; and in media containing copper. The uniform result of these tests was that the germ tubes near the perforations grew towards the opening *if it communicated with a layer of media in which no spores had been placed.* The presence or absence of copper was not found to have any influence on the *direction* of growth. The hyphae grew towards, and in cases where the concentration of copper was not too great, grew through the perforation into the copper-containing medium. *Fig. 6* illustrates such a case. The spores of Rhizopus were sown in a rich nutrient agar. Upon germination they immediately turned towards a perforation in the mica plate which communicated with a non-nutrient agar impregnated with 0.005 *n* $CuSO_4$.

Finally, I prepared a series of cultures in large Van Tieghem cells 5^{cm} in diameter. The nutrient agar in which the spores were placed occupied the part marked *a* in *fig. 7*. The portions of agar marked *b b* were prepared from nutrient or non-nutrien agar to which varying concentrations of copper had been added The portions *c c* are checks, being duplicates of *b b* without the copper. The inoculation was made by placing a few spores of Rhizopus at the center of *a*. The cultures were then placed in the incubator at 28° C. until the fungus had made considerable growth. The cells were then opened and *b b* and *c c* were pushed towards *a* until in slight contact and occupying the positions *b'b'* and *c'c'*. More or less of the Cu in solution in *b'* and *b'* would then pass into *a*, and if the fungus was either attracted or repelled by the Cu it would be readily determined by watching the behavior of the hyphae as they grew down *a* towards the extremities *dd* between the copper-containing portion *b′* and the check preparation *c′*. The hyphae did curve towards *b′* and *b′*, but also towards *c′* and *c′*, and the *larger* the portions *b′* or *c′*, the more strongly they curved towards them. This curving took place even when *b* and *c* contained no nutrient substance, the hyphae growing from the nutrient portion *a* into a nonnutrient medium. Cultures on this plan were repeated many times and varied in many ways, *e. g.*, varying the consistence of the agar, the nutrients used, the concentration of copper, etc.

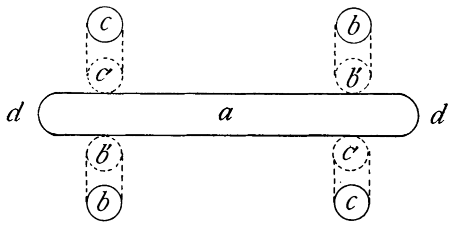

FIG. 7.

My interpretation of these results is that Rhizopus is markedly negatively chemotactic to some secretion of its own mycelium, and this negative chemotropism is much greater than any positive chemotropism it may have for food substances or oxygen. This method then, which seems to be satisfactory, has failed to show that Rhizopus has positive or negative chemotropism towards copper or allied metals. These cultures were used to a less extent with several other saprophytic and two parasitic forms, with the same result in regard to chemotropism towards copper. A large number of photomicrographs and drawings with camera lucida were made, but those reproduced in *figs. 5* and *6* are as instructive as any.

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